Pestic. Sci. 1996, **46**, 49–53

Sterols that Modify Moulting in Insects*

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(Received 3 April 1995; revised version received 30 May 1995; accepted 12 September 1995)

Abstract: The development of insects is under the control of a steroid hormone, ecdysone. This paper reviews suicide substrate type inactivators designed to inhibit the biosynthesis of ecdysone. Several series of acetylenic derivatives of cholesterol were synthesized and their biological effects on the prothoracic glands of the locust were investigated *in vitro*, enabling structure–activity relationships to be studied.

Information on the ability of brassinosteroids, a series of plant growth regulators, to inhibit ecdysteroid activities of insects is discussed as a precursor to a study of brassinosteroid ecdysone mimics.

Key words: suicide-inhibitors, irreversible inactivators, insect moult, moult inhibition, ecdysone biosynthesis, *Locusta migratoria*.

1 INTRODUCTION

A major characteristic of insect development is the process of the regular shedding of the old cuticle and the synthesis of a new one. This process, known as moulting, is controlled by the steroid hormone ecdysone (Fig. 1, **1**).[1] Ecdysone is synthesized during post-embryonic development in the endocrine glands, referred to as 'prothoracic glands' in the case of insects.[2] Reproductively competent female insects also synthesize ecdysone in their ovaries.[3] Ecdysone is usually considered to be a prohormone, as it is hydroxylated to 20-hydroxyecdysone (Fig. 1, **2**), the major moulting hormone. For fundamental studies, as well as applied research, we wish to modify the development of insects at the site of action of the moulting hormone for which purpose selective and irreversible inhibitors of ecdysone biosynthesis, as well as substances which are able to antagonize the effects of ecdysteroids, have been designed.

2 INHIBITORS OF ECDYSONE BIOSYNTHESIS

Suicide substrate inhibitors[4] are the most appropriate materials to inhibit ecdysone biosynthesis since they are very selective, being presumed to interact with the active site of the appropriate enzymes. They should have a long-lasting effect as a result of forming a covalent bond at the active site of the enzymes. To design such inhibitors, the exact molecular structure of the natural substrates, or the biosynthetic intermediates selected as targets must first be elucidated, having first characterized the enzymes which are implicated. The biosynthetic pathway of ecdysone arrived at by elucidating the structure of biosynthetic precursors, and the enzymes which are responsible for their formation, are shown in Fig. 2; until now, only the final steps in this pathway had been determined.

Starting with cholesterol, which the insects obtain by dealkylating phytosterols, the pathway consists of

* Based on a paper presented at the meeting 'Chirality in crop protection chemistry' organised by the Pesticide Group of the SCI and held at 14/15 Belgrave Square, London SWIX SPS on 7 March 1995.

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Pestic. Sci. 0031-613X/96/$09.00

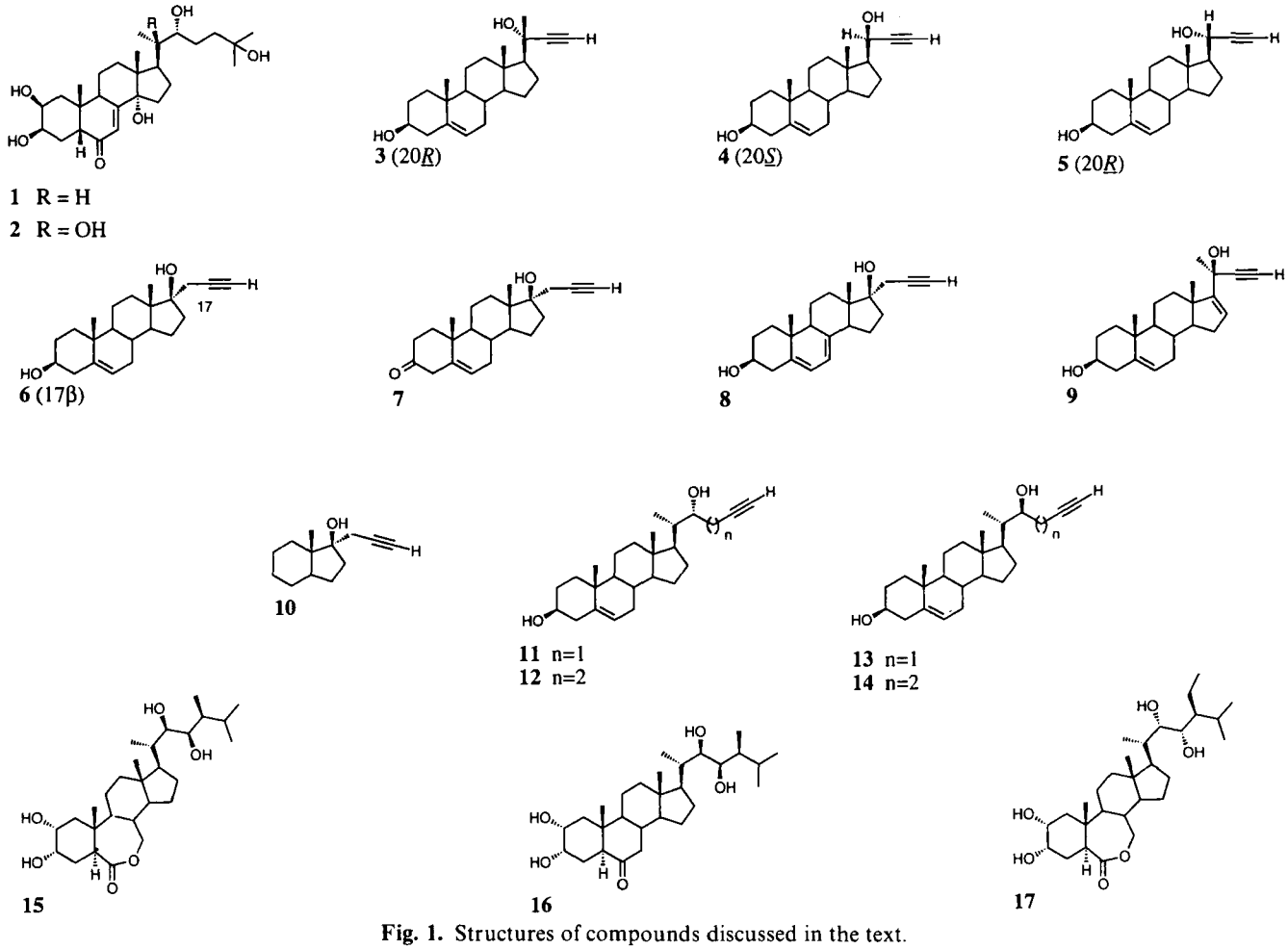


Fig. 1. Structures of compounds discussed in the text.

sequential hydroxylation of 2,22,25-trideoxyecdysone at C-25, C-22 and, finally, at C-2.[5,6]

Since 2-deoxyecdysone expresses some hormonal activity,[7] we decided to target inhibition of hydroxylation of its two precursors, the 2,22,25-trideoxy- and 2,22-dideoxy compounds, rather than this 2-deoxy derivative.

The enzymes responsible for these two hydroxylations are cytochrome P-450-dependent monooxygenases[8] and it is well established that such enzymes can be inactivated by means of suicide substrates such as acetylenic derivatives.[4]

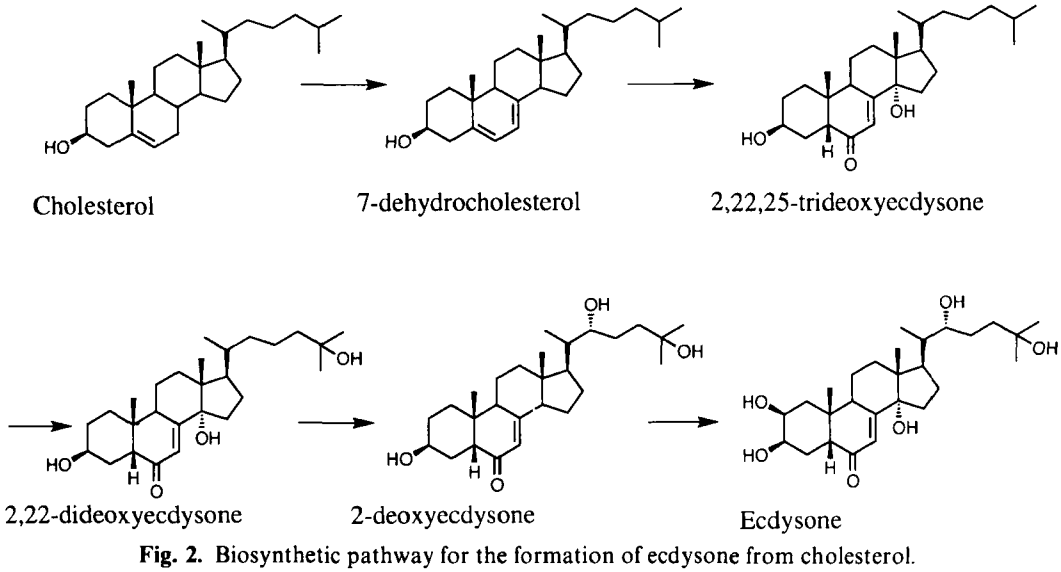


Fig. 2. Biosynthetic pathway for the formation of ecdysone from cholesterol.

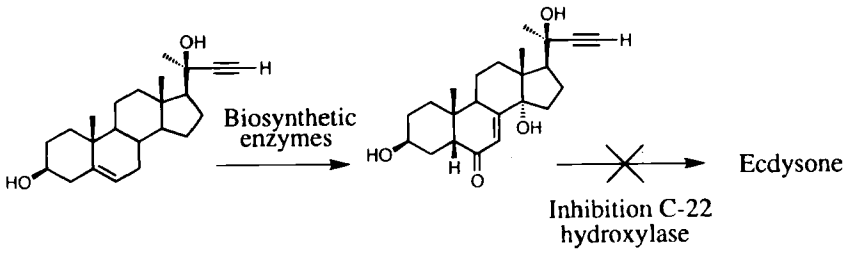


Fig. 3. Cholesterol nucleus inhibitors were transformed to ecdysteroid inhibitors before inhibiting C-22 hydroxylase.

3 ACETYLENIC INHIBITORS OF C-22 HYDROXYLASE OF ECDYSONE BIOSYNTHESIS

Inhibition is presumed to take place in the prothoracic glands, where cholesterol is transformed to ecdysteroid so that the cholesterol nucleus can be used to carry the inhibitory acetylenic function instead of the ecdysteroid moiety. Indeed, one can assume that the cholesterol nucleus can be transformed into an ecdysteroid precursor by the biosynthetic enzymes before reaching its target, the C-22 hydroxylase, which will then be inactivated by the acetylenic function (Fig. 3).[9]

Two series of compounds were first synthesized[9] (Fig. 4) and tested for their potential inhibitory effect on ecdysone biosynthesis in the prothoracic glands of *Locusta migratoria migratorioides* Reiche & Fairm.[10] They have in common the cholesterol nucleus and a side chain bearing an acetylenic group. Several types of side chain have been synthesized ranging from the simple acetylenic group at C-22 to the complete side chain of cholesterol or to a side chain similar to that of ecdysone with an hydroxyl group at C-25. These two series of putative inhibitors, A and B differ only in the presence of an additional hydroxyl at C-20 in series B (Fig. 4).

Compounds of series B which have an *S* C-20 hydroxyl group are better inhibitors of ecdysone synthesis than their A counterparts; for example, the most potent inhibitors, B1 and B6, reduced ecdysone production by 60% at 10^{-4} M and significant inhibition was observed at 10^{-7} M.

Investigation of the mechanism of action at the molecular level was not possible as the purified enzymes involved in ecdysone biosynthesis were not available. However, prothoracic glands pre-incubated with these inhibitors never recover the normal values of ecdysone production obtained in the absence of inhibitors, so that one can assume that they are irreversible inhibitors.[11]

The selectivity of their inhibition is illustrated by conversion studies with radio-labelled precursors. Using these precursors, it was shown that these inhibitors essentially depress hydroxylation at C-22; indeed, in the presence of the inhibitor-treated glands labelled 2,22-dideoxyecdysone was converted to 22-deoxyecdysone in high amount.[11]

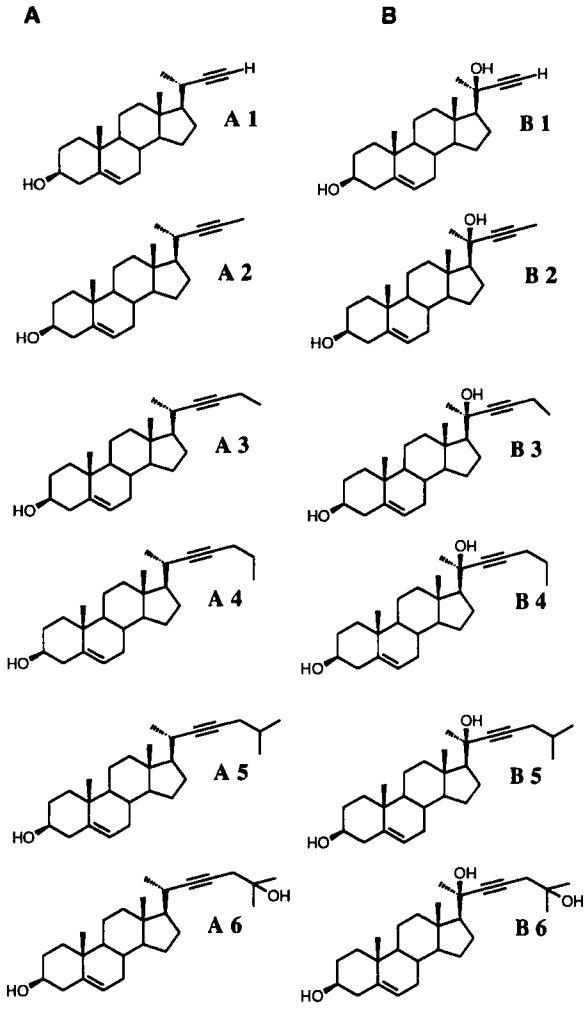


Fig. 4. Putative synthetic inhibitors of ecdysone biosynthesis. Series A; without hydroxyl group at C-20. Series B; with hydroxyl group at C-20.

4 REFINEMENT OF THE CHEMICAL STRUCTURE OF ACETYLENIC INHIBITORS OF C-22 HYDROXYLASE OF ECDYSONE BIOSYNTHESIS; A PROBLEM OF STEREOCHEMISTRY

Of the two potent inhibitors, B1 and B6, B1 possesses the better biological profile as B6 induces a cytotoxic effect. B1 was therefore selected as lead compound and attempts were made to improve its efficacy by modifying its structure. By inverting the stereochemistry at

TABLE 1
In vitro Inhibitory Effect (%) of Various Synthetic Compounds on Ecdysone Biosynthesis in Prothoracic glands of *Locusta migratoria*

Compound[a]	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
4	60	60	45	27	—
5	36	0	—	—	—
6	82	75	65	45	25
7	86	53	0		
8	75	–	15	0	
9	94	69	32	0	
10	50	0			

[a] See Fig. 1 and text.

C-20 of B1 (*S*, 20-OH), we obtained compound **3** (*R*, 20-OH; Fig. 1) which had no inhibitory activity in our bioassay.[12]

This finding indicates a role for the hydroxyl group at C-20 and led us to synthesize compounds **4** (*S*, 20-OH) and **5** (*R*, 20-OH)[13] in which the methyl at C-20 was removed and replaced by hydrogen. We assumed that these secondary propargylic alcohols would react more readily with the active site of the enzyme, leading to more efficient inhibition.

Compound **4**, with a less hindered hydroxyl group, was a much more potent inhibitor *in vitro* than **5** (Table 1).

The role of the hydroxyl group in the vicinity of the triple bond was illustrated by another study in which the hydroxyl group in **4** was shifted from *S*, C-20 to C-17β giving compound **6** (17β) which is, at present, our most potent inhibitor (Table 1). It depresses ecdysone production *in vitro* by 25% at 10^{-8} M. Given orally, **6** inhibited the development of *Drosophila melanogaster* Meig. *in vivo*. Any minor modification of the sterol nucleus markedly changed the inhibitory effect of the putative inhibitor. Indeed, the transformation of 3β-OH to 3-C=O led to **7**, which is only slightly inhibitory. The introduction of a double bond at C-7 (compound **8**) or at C-16 (compound **9**) also reduced the inhibitory effect. Finally, with the aim of investigating the importance of molecular size in order to determine the minimal structure required to inhibit ecdysone biosynthesis, we synthesized the bicyclic compound **10** using a well-known procedure.[14,15] This compound which contains the structure of the C and D rings of **6** was inhibitory only at 10^{-4} M, i.e. it is four orders of magnitude less effective than **6** (Table 1).

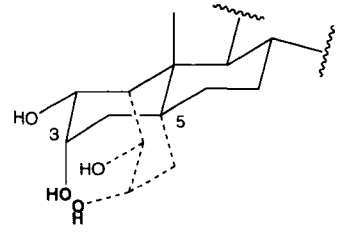
5 ACETYLENIC COMPOUNDS INHIBITORS OF C-25 HYDROXYLASE OF ECDYSONE BIOSYNTHESIS

To test the generality of our working hypothesis, we targetted inhibition of C-25 hydroxylation in ecdysone biosynthesis which can be achieved by extending the acetylenic side chain. Thus two series of compounds differing in the length of the side chain, which contains one (**11** and **13**) or two (**12** and **14**) carbon atoms after C-22, were synthesised.[15] They differ also in the stereochemistry at C-22. Our bioassay showed that inhibition increases with side chain length (see compounds **11–14** in Fig. 1 and Table 1). This inhibition is also more marked when the configuration at C-22 is *R*, which is that of natural ecdysone.

6 INHIBITORS OF ECDYSTEROID BIOLOGICAL ACTIVITY. 'BRASSINOSTEROID-LIKE' SUBSTANCES

Brassinolide (**15**), a polyoxygenated phytosterol and a potent plant growth regulator, is able to promote cell elongation and division in several plants;[16] its structure was established to be (22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxo-5α-cholestan-6-one (**15**) using X-ray techniques.[17] Soon after its isolation in 1979, many related compounds, either naturally occurring or synthetic, have been reported;[18] at present, about 30 naturally occurring brassinosteroids have been isolated and their biological activities have been investigated in different biological systems.[18]

These brassinosteroids exhibit structural similarities with ecdysteroids in that they both contain the entire cholesterol skeleton with the complete side chain. The existence of two hydroxyl groups in the A ring and two in the side chain makes these compounds very soluble in water. Although ecdysteroids contain a *cis* and the brassinosteroid a *trans* A/B ring junction, 3β-OH in ecdysteroids and 3α-OH in brassinosteroids occupy the same site (Fig. 5). For this reason, it was decided to investigate the effect of brassinosteroids on the moulting process of insects. Two of the seven brassinosteroids investigated induced the evagination of imaginal disc of a Dipteran, *Phormia terra-novae* (R.-D.) while the others compete with the 20-hydroxyecdysone (**2**).[19] This experiment clearly demonstrated that brassinosteroid-like compounds can interfere with the ecdysteroid



—— Brassinosteroid partial structure
----- Ecdysteroid partial structure

Fig. 5. Comparison of A and B rings of brassinosteroids and of ecdysteroids.

hormone system of insects. In particular, castasterone (**16**) and 22*S*,23*S*-homobrassinolide (**17**) exhibit high affinity to the ecdysteroids receptor.

We are at present exploring the potentialities of using brassinosteroids analogues as anti-hormones, our objective being the investigation of the ecdysteroid receptor as well as the mode of action of the hormone. Work is in progress to elucidate this interaction.

7 CONCLUSION

In principle, it is possible to modulate the development of most insects at the site of action of moulting hormones by using either synthetic inhibitors or natural substances. The designing of synthetic inhibitors opens new areas of research. They can be used to investigate the biology of the moulting process as well as having practical applications. However in regard to the latter, much work is needed to elucidate how these rather large lipid-type molecules penetrate to the site of action in insects under natural conditions.

ACKNOWLEDGEMENT

Financial support of this work by Ciba-Geigy is gratefully acknowledged.

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